

“Research-grade” Targeted Proteomics Assay Development: PRMs for PTM Studies with Skyline

or,

“How I learned to ditch the triple quad and love the QE”

Jacob D. Jaffe

Skyline Webinar July 2015

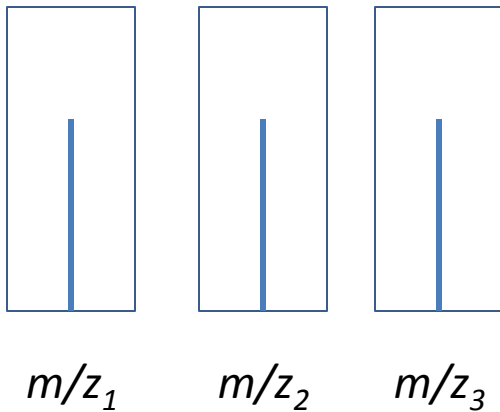
Outline

- Definitions
- When do PRM assays make sense?
- Considerations for PRM method development
- Live Demo

Definition of PRM

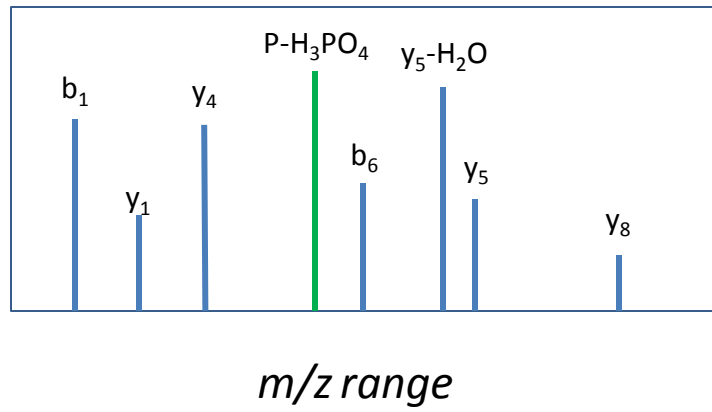
- PRM = MRM-HR = HR-MRM = Targeted Full Scan MS/MS
- Closest spiritual cousin is triple-quad based MRM/SRM, but:

SRM
Q3 mass analyzer



Discrete transitions,
hardware selected

PRM
mass analyzer



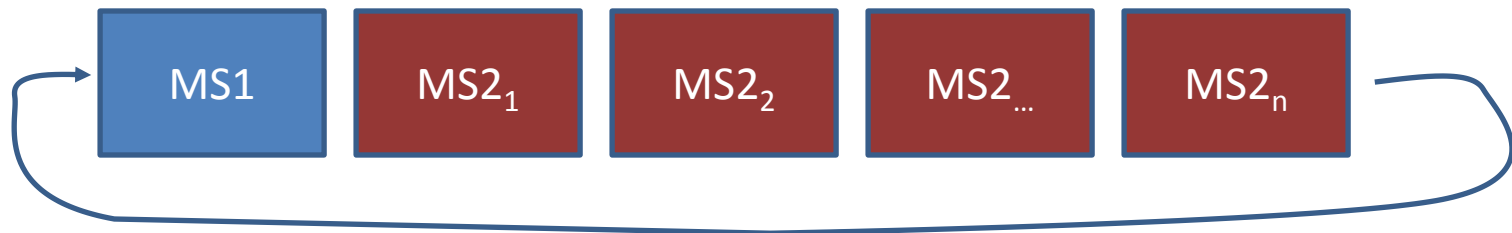
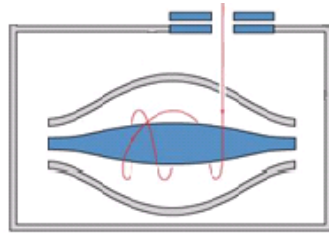
Full scan

Definition, continued

- Assay is completely deterministic
- Precursor m/z (list) is specified
 - Possibly scheduled
 - Quadrupole or ion trap selection/isolation
- Fragmentation is performed
 - Any kind is OK
- Full MS/MS spectrum is recorded
 - Any analyzer: Orbitrap, TOF, scanning quad, ion trap, etc.
- Usually a full scan MS spectrum is also periodically recorded
 - Two chances to verify and quantify!

Common configuration: high resolution mass analyzer

- Orbitrap or TOF



- Precursor cycle vs. Acquisition loop cycle
 - Precursor cycle: time it takes to loop through precursor list
 - May vary during method
 - Governs points across peak
 - Acquisition loop cycle: Time from full scan to full scan with intervening # of MS/MS
 - May affect instrument performance, full scan points across peak

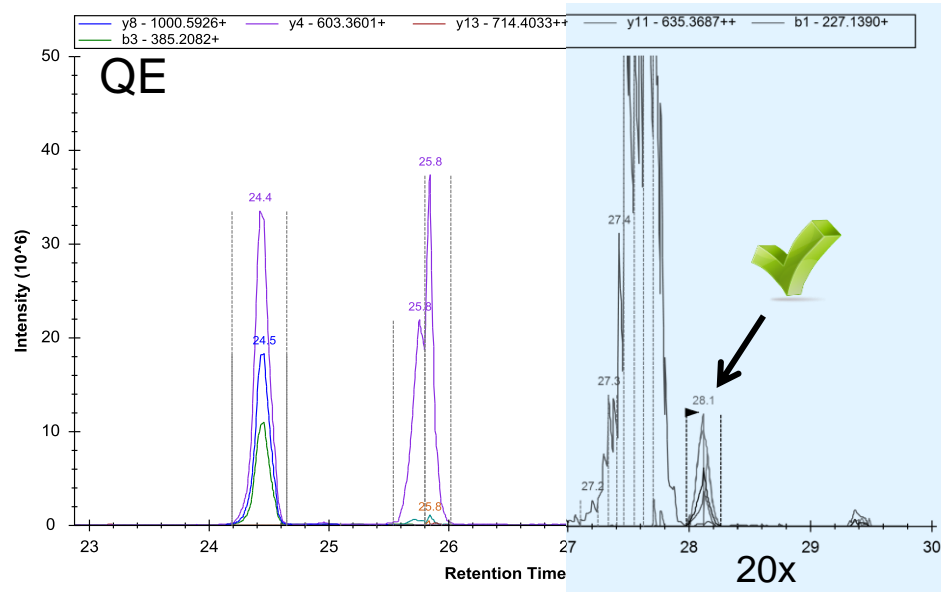
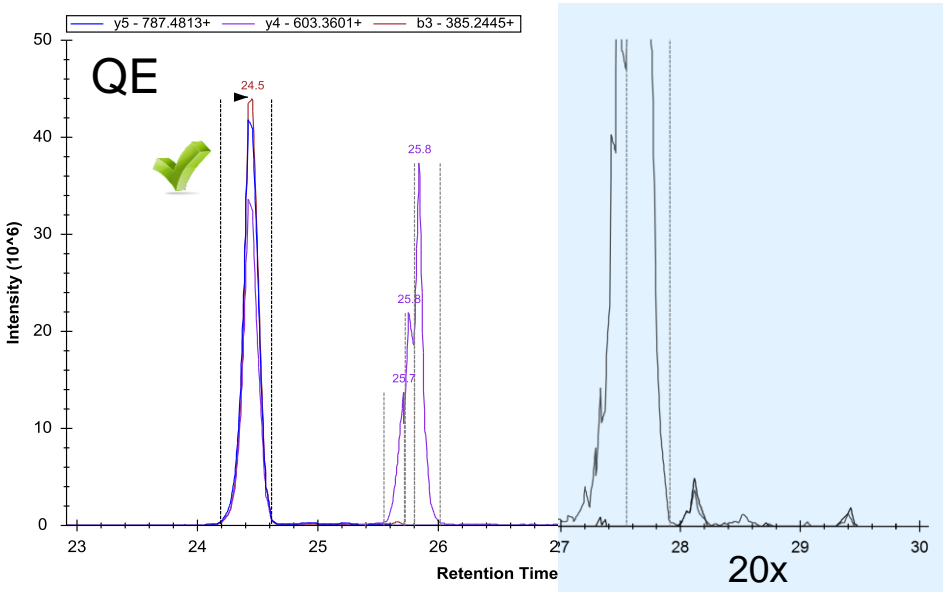
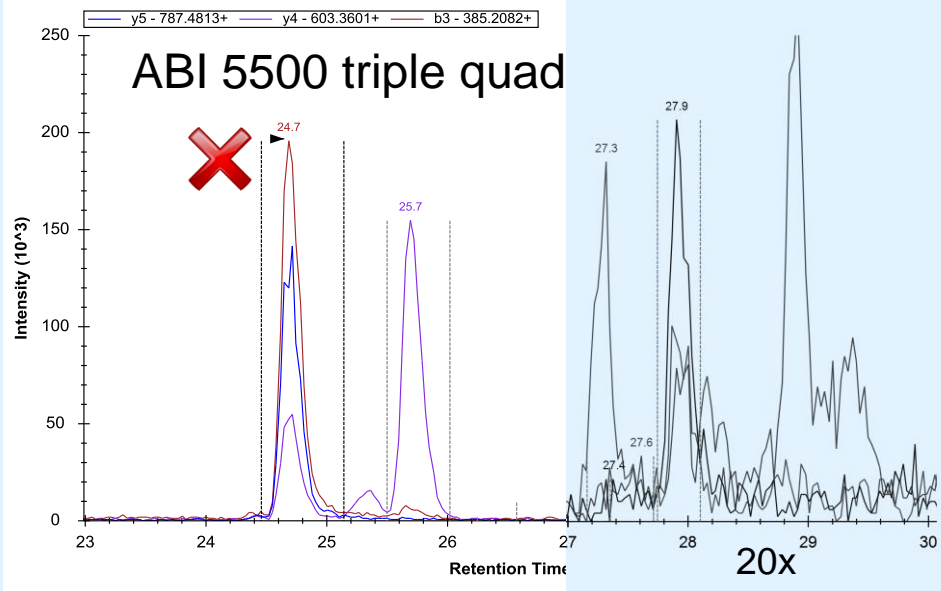
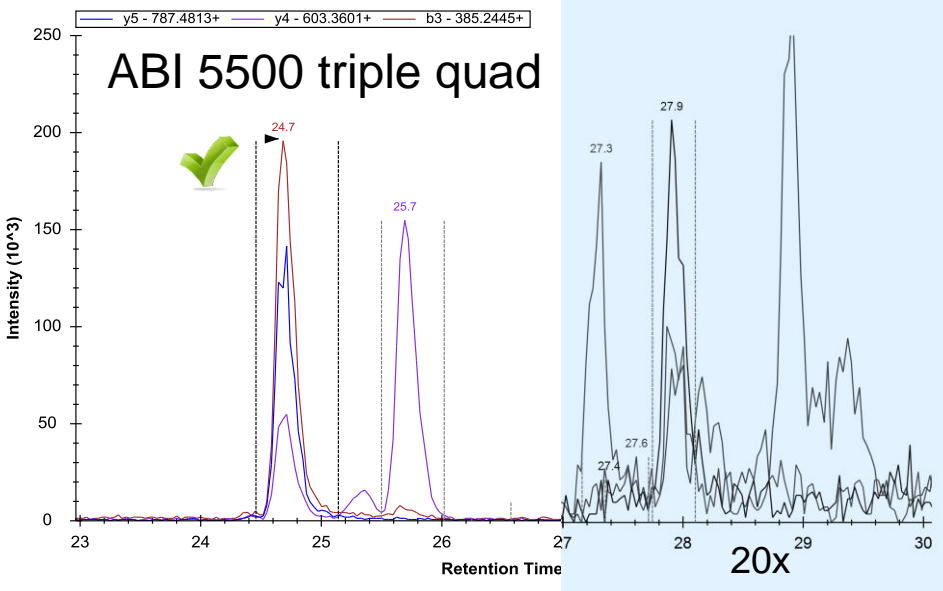
When do PRMs make sense?

- Exquisite selectivity required
 - Unit (quadrupole) vs. ppm (hi-res)
- Post-translational modification localization is required
 - GVDQ(pS)PLTPAGGK vs. GVDQSPL(pT)PAGGK
- Rapidly convert discovery data to targeted assay
 - Stay within platform
- You don't have a triple quad!
 - But still want the benefits of targeted proteomics

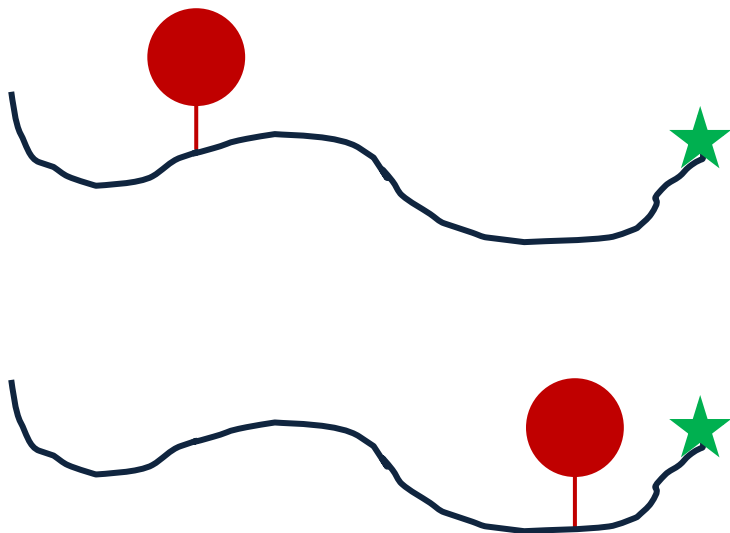
High resolution adds value to selectivity

K_{me3}SAPATGGVK_{pr}K_{pr}PHR₁₀ m/z 551.9940 z=3

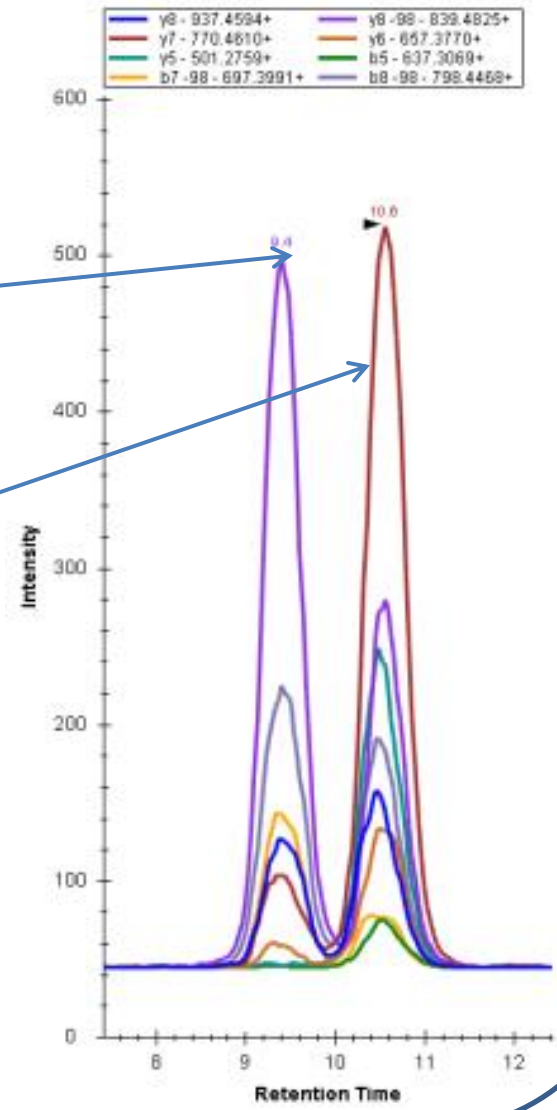
K_{ac}SAPATGGVK_{pr}K_{pr}PHR₁₀ m/z 551.9819 z=3



PTM Localization – shared ions, differential ions



Differentially Phosphorylated Peptides
With Same Base Sequence

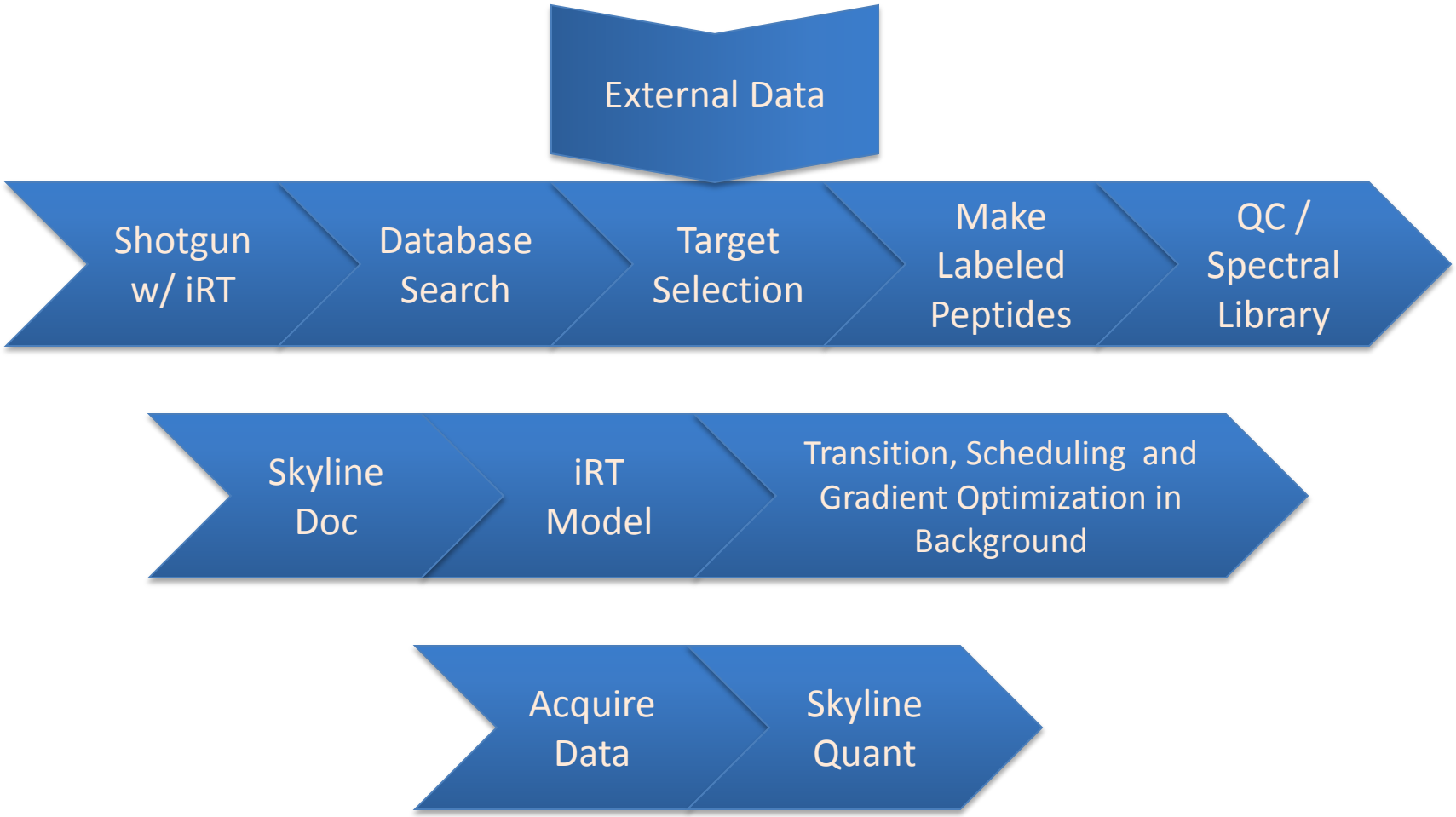


Discovery Proteomics to PRM – Short version / Label Free



* iRT peptides recommended

Discovery Proteomics to PRM – Long version



Planning ahead for success

- Strongly consider including iRT peptides in every single sample you run in your lab
 - Diverse retention times, well spaced
 - High enough levels to trigger MS/MS
 - Or, include targeted scans
 - Or, determine RTs with precursor quant in Skyline
 - This can also be very beneficial for scheduling tight windows
- Use a search engine supported by Skyline spectral library import
- Set up your funky PTMs in advance in your document
- Learn about Skyline's secret PTM notation for import

Document refinement

- Keep a lot of transitions around initially
 - You can always get rid of them later
 - You can take them from the spectral libraries
 - In theory: the more transitions, the more signal-to-noise
 - Also in theory more sensitive than MRM, but generally not in practice
- Take advantage of the raw data spectrum viewer functionalities
 - Helpful for both MS and MS/MS inspection
- Use that high res!
 - Narrow your import m/z tolerances
 - Inspect the ppm errors

The all important dotp

- dotp = dot product
 - Metric of observed transition relative intensities in comparison with spectral library example
- Better than a search engine score!
 - Expect > 0.9 under most circumstances
- Extremely useful in differentiating among similar analytes
- Spectral library quality important
 - Garbage in, garbage out

Standardization Considerations

- Label free
 - Requires high degree of system reproducibility
 - Hard to compare samples longitudinally
- Synthetic peptides
 - Highest degree of rigor
 - Highest cost in time, \$
 - More optimization required
- SILAC
 - Increases complexity, chance for interference
 - Standard is “prenormalized”
 - Consider growing up a vat of standard for longitudinal performance
- Chemical labels? (+ standards?)

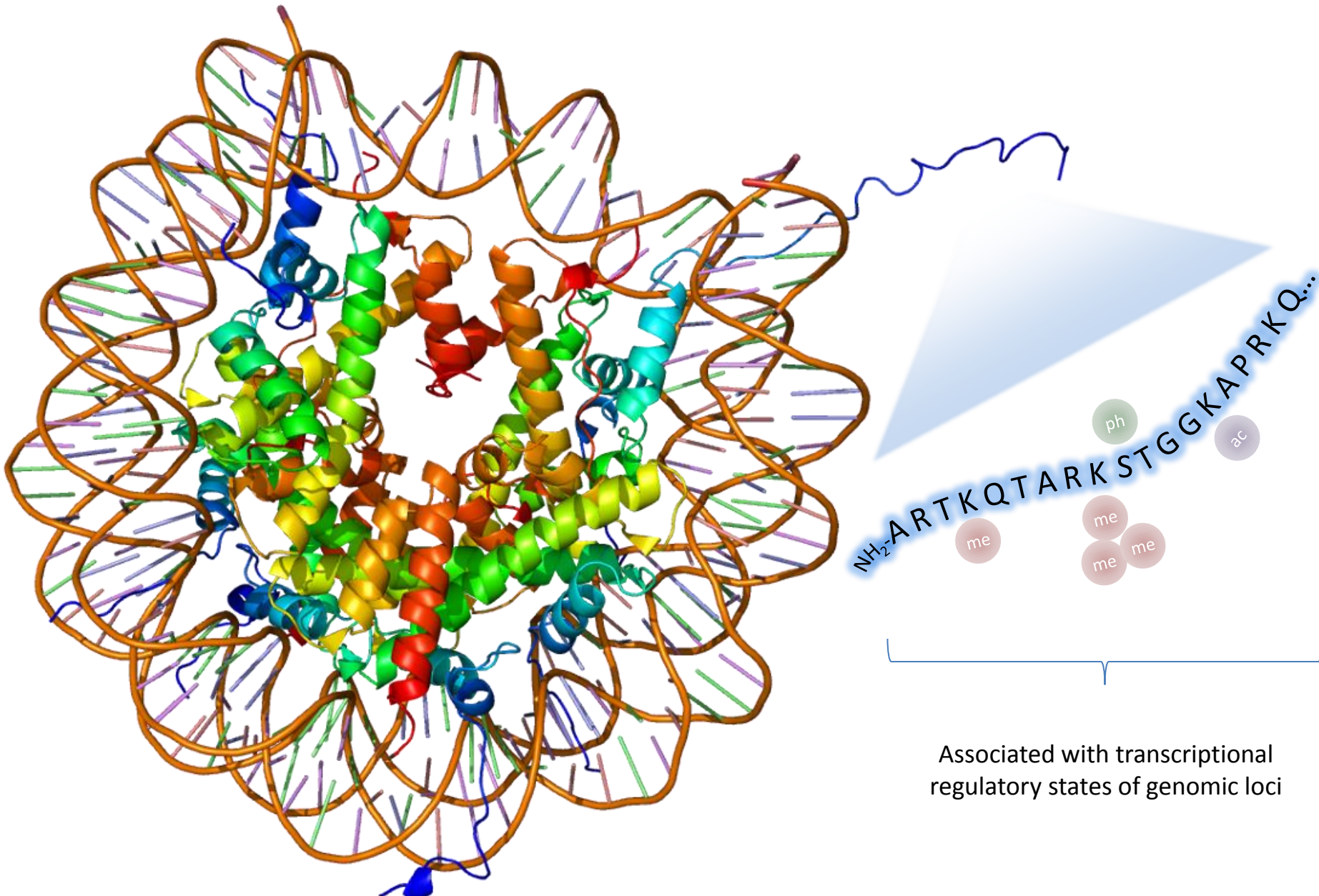
Data analysis considerations

- Be patient, use all metrics at your disposal
- Consider time window import limits
 - But relies on RT or other indicators in spectral library / RT models
- Consider further minimizing your document when happy with data
 - Hi-res data, skyd files get big

“Research Grade” PRM Concept

- A quantitative, targeted proteomics assay suitable for “everyday” use
- Ideally standardized with synthetic peptides (or SILAC)
- Rapid design cycle using discovery data/platform
- Enables longitudinal comparisons across days, months, years
- Output useful for rapidly guiding biology
- NOT:
 - Obsessed with LOD/LOQ
 - Suitable for clinical deployment

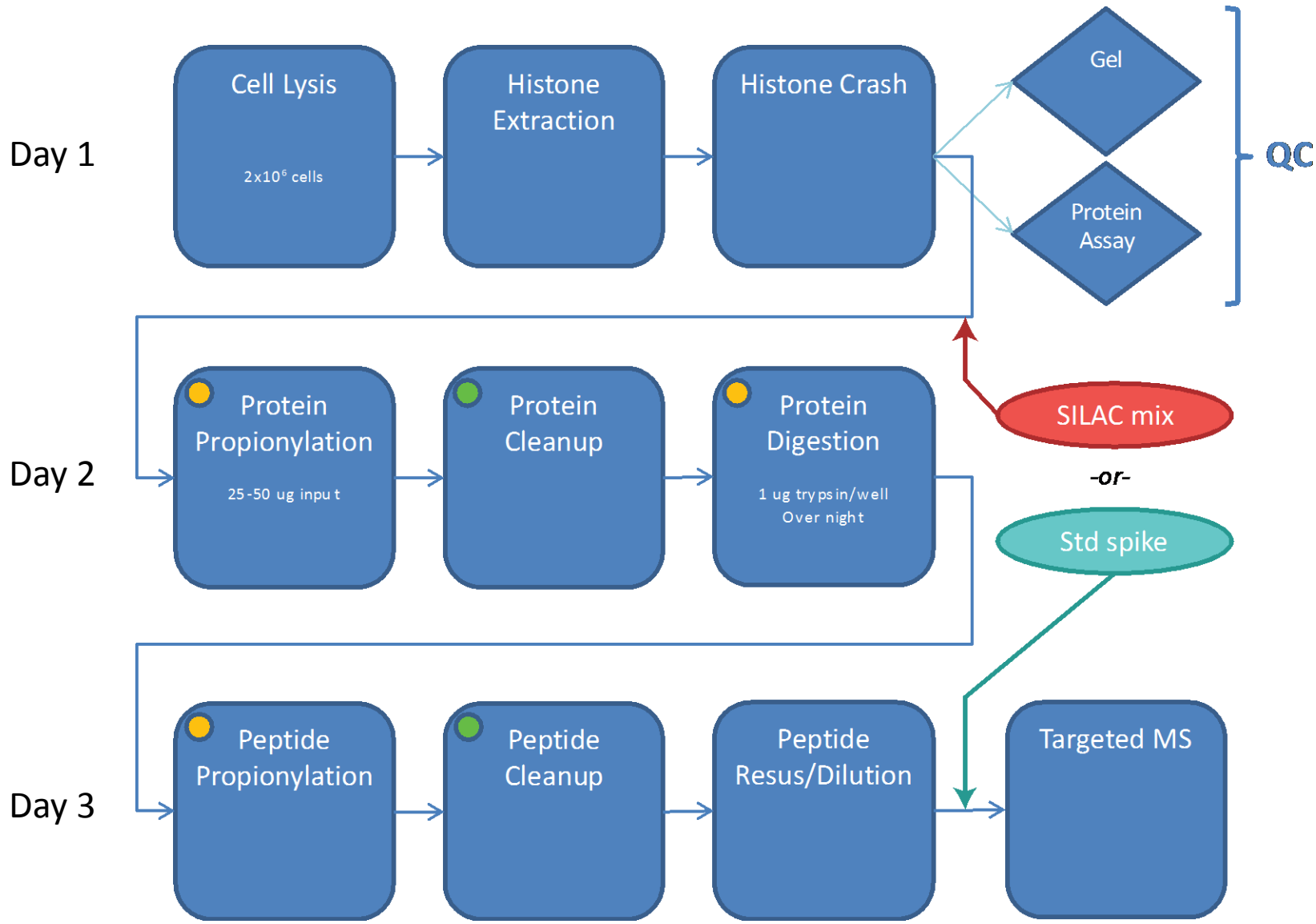
Histones and their post-translational modifications



Associated with transcriptional regulatory states of genomic loci

Source: PDB 1AOI, Luger et al. *Nature* (1997)

Sample preparation process and standardization



● Agilent Bravo LH – fully automated

● 96-well SPE – semi-automated